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A BASIC FUCHSIN AND ALKALINIZED METHYLENE BLUE RAPID STAIN FOR EPOXY-EMBEDDED TISSUE[1]

JAMES D. HUBER, FRANK PARKER, and GEORGE F. ODLAND,
Departments of Medicine and Biological Structure,
University of Washington School of Medicine,
Seattle, Washington 98105

ABSTRACT. **Sections 1 μ thick of epoxy-embedded, OsO_4-fixed tissues were stained with 4% aqueous basic fuchsin at 70 C for 1 min, rinsed well and destained, also at 70 C, for 1 min. A 2% aqueous methylene blue solution, alkalinized to pH 12.5 by mixing 1 *N* NaOH with the dye on the slide in the proportion of about 2:1, was then allowed to act for 2 min at 25–27 C. The stain was rinsed off the slide, and the preparation air dried before applying a mounting medium and cover glass. The mounting medium consisted of immersion oil sealed with epoxy household cement. Stains had not faded after 1 yr. The method is simple, rapid (total time 4–5 min), and provides sharp contrast between cellular and connective tissue components.**

Methods for staining 1 μ thick sections from OsO_4-fixed, plastic-embedded tissue originally employed single or several closely related basic dyes (Trump *et al.* 1961; Winkelstein *et al.* 1963; Richardson *et al.* 1960; Munger 1961). The techniques for single dyes effect a monochromatic staining which lacks the desired contrast to show clearly nuclear, cytoplasmic, and extracellular components of the tissue; therefore, some investigators have found that two or more dyes provide greater differentiation between tissue constituents (Munger 1961; Grimley 1964; Grimley *et al.* 1965). Disadvantages in the published methods are: (1) considerable time is often required for completing the staining (Munger 1961; Grimley 1964, 1965); and (2) removal of the embedding medium is occasionally required to obtain adequate staining (Munger 1961).

The present communication reports a rapid staining method in which basic fuchsin and methylene blue provide vivid contrast between the cellular and connective tissue elements. No removal of the embedding medium is required and an oil mounting sealed by epoxy resin prevents the stain from fading for at least 1 yr.

MATERIAL AND METHODS

Preparation of Tissue Sections

Samples of human skin and nerve, and rabbit skin, kidney, blood vessel, cartilage, and muscle (1–3 mm^3) were fixed for 1–2 hr in 2% OsO_4 buffered with *s*-collidine. Some tissues were also fixed in glutaraldehyde alone or glutaraldehyde followed by OsO_4, and in conventional combinations of OsO_4

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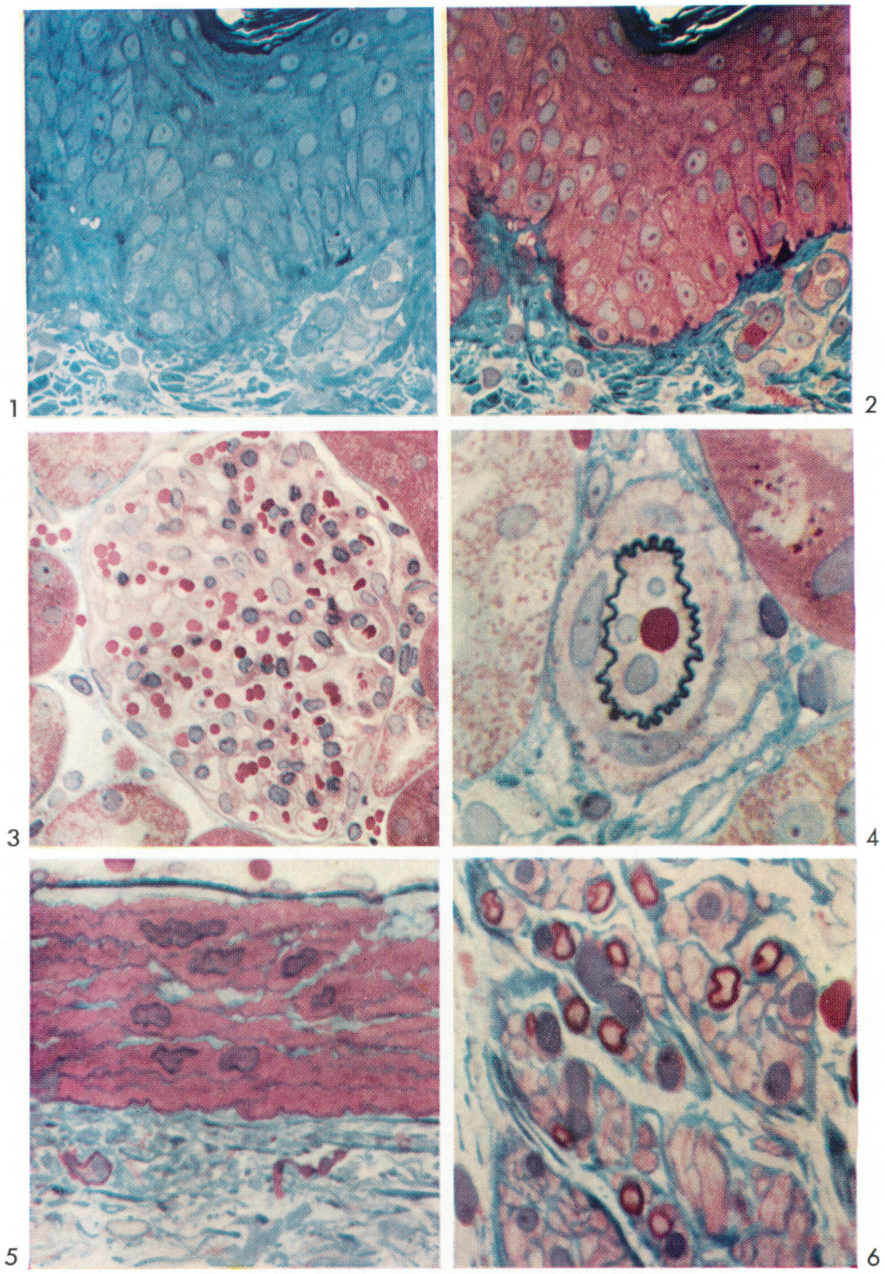

FIGS. 1 and 2. Adjacent sections of human skin stained with the Richardson azure II-methylene blue technique (Fig. 1) and the dibasic method (Fig. 2). Connective tissue in the dermis (blue) is differentiated from the cellular elements (pink with purple nuclei) by the dibasic method (Fig. 2). Two small blood vessels (lower right) are seen in the dermal papillae. The cells of vascular wall stand out clearly in pink against the blue collagen. × 360

FIG. 3. Rabbit glomerulus with surrounding renal tubules stained by the new dibasic method. Red blood cells stain red; nuclei, purple; and mitochondria of tubular cells appear as a more intense red color than their surrounding cytoplasm. Collagen is blue. × 360

with other buffers: veronal-acetate, phosphate, and bicarbonate. Fixed specimens were dehydrated in increasing concentrations of alcohol and embedded in Epon (Luft 1961). Sections 1 μ thick were picked up in a small quantity of water with a wire loop and placed directly on clean glass slides and allowed to dry on a hot plate at 70 C prior to staining.

Dye solutions

A. Basic fuchsin, Cert. No. NF-81, 4% in distilled water; dissolved at 70 C and held at this temperature during staining. The solution must be prepared fresh each day to avoid precipitates.

B. Methylene blue, 2% in distilled water. Dye used: USP powder for prescription compounding; Mallinckrodt Chemical Works.

Staining procedure

1. A few drops of staining solution A is placed on the sections and allowed to remain for 1 min, with the slide on a hot plate at 70 C.
2. The slide is rinsed with distilled water to remove all excess dye, replaced on the hot plate, and destained with a few drops of distilled water for 1 min.
3. The excess water is removed by blotting with filter paper and the slide allowed to dry at room temperature with the aid of a jet of compressed air.
4. Two drops of 0.1 *N* aqueous NaOH are applied and followed immediately by 1 drop of solution B. The slide is agitated for 15 sec to mix the solutions and staining allowed to continue for 2 min. The required staining time may vary with the tissue and the results desired.
5. The staining reagents are quickly rinsed off the slide with distilled water and the sections allowed to dry.
6. Cover slips are mounted after a drop of immersion oil (Zeiss) has been placed on the sections. Small amounts of epoxy household cement are used to seal the cover slip and allowed to harden overnight at room temperature, or 1–2 hr in a 60 C oven.

Results

Characteristic colors obtained with this staining technique on human and rabbit tissues are shown in figures 1 and 3–6. Figures 1 and 2 are photo-

Fig. 4. Cross section through a small renal arteriole from a rabbit (dibasic stain). The elastic lamina takes a heavy blue stain. A single red blood cell is seen in the lumen. Small amounts of blue collagen are seen between adjacent renal tubules. × 1090

Fig. 5. Portion of a rabbit coronary artery in cross-section (dibasic stain). The internal elastic lamina (top) has an intense blue color, while the smooth muscle cells of the media are pink with purple nuclei. Blue-stained connective tissue is seen between the muscle cells and predominates in the adventitia. Occasional pink-staining fibroblasts are seen amid the collagen in the adventitia. × 735

Fig. 6. Cross section through a human sural nerve (dibasic stain). Myelin is stained brilliant red; axoplasm, pale orange. Schwann cell nuclei are purple; their cytoplasm, pink. Surrounding collagen is blue. × 860

micrographs of adjacent sections of human skin which compare the present staining method (figure 2) with the Richardson (1960) technique (figure 1). Greater color contrast between cellular and connective tissue elements is achieved with the dibasic stain. The collagen of the dermis stains a brilliant blue, readily distinguished from the pink hues of the cytoplasm in epidermal, vascular, and connective tissue cells. Cell nuclei, which are purple, are clearly differentiated from the cytoplasmic portions of cells.

A rabbit glomerulus with portions of surrounding renal tubules is pictured in figure 3. Capillary loops are readily outlined and many contain pink-staining red blood cells. The basement membrane is not clearly distinguished, but a few blue-staining collagen fibers can be seen between the glomerulus and tubules. Mitochondria within the tubular cells have a more intense red hue than the surrounding cellular cytoplasm.

A renal arteriole and a portion of a coronary artery are shown in figures 4 and 5, respectively. In both, the elastic lamina appears as a distinct blue line. The pink-staining endothelial and smooth muscle cells contrast clearly with the blue-staining connective tissue found between the muscle cells, as well as within the tunica adventitia of the coronary artery.

Figure 6 shows a cross section through a portion of a human sural nerve. Myelin is brilliant red within the lighter pink cytoplasm of the Schwann cells; axoplasm is salmon pink. The perineural connective tissue contrasts clearly in deep blue.

Cartilage (not illustrated) appears as a blue-green matrix with pink-staining chondroblasts. Mast cell granules assume both vivid red and blue colors. Examination of human xanthomas, which retain appreciable amounts of lipid despite the embedding procedure, displays macrophages containing bright, red-stained droplets of lipid.

In all the tissues examined, the staining reactions appeared to be the same regardless of the fixatives or buffers used. No alterations in the staining time were required because of the use of different fixatives or buffers.

Discussion

The staining time of 1 min with basic fuchsin and subsequent 1 min destaining was found to be optimal for a variety of tissues.

Methylene blue requires the presence of 2 parts of 0.1 *N* NaOH to provide a pH of about 12.5. This degree of alkalinity is apparently needed to cause the methylene blue to penetrate the embedding resin, as well as to fix the stain to various tissue components. More concentrated solutions of NaOH loosen the sections from the glass slides. The alkali and methylene blue must be mixed immediately on use because precipitates form within 30 min. Staining time for the methylene blue may be varied to suit it to the tissue, the thickness of the sections, and the intensity of color desired.

Sealed-oil type mounting was found to be useful since no observed color fading has occurred in slides kept for at least a year. When Epon epoxy resin is used as the mountant, significant fading occurs. The use of Zeiss im-

mersion oil has the added advantage of providing an ideal refractive index (1.5150).

The rapidity and ease with which the staining method is accomplished should make it suitable for routine use and photomicrography.

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